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spa Typing Method for Discriminating among *Staphylococcus aureus* Isolates: Implications for Use of a Single Marker To Detect Genetic Micro- and Macrovariation

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Strain typing of microbial pathogens has two major aims: (i) to index genetic microvariation for use in outbreak investigations and (ii) to index genetic macrovariation for use in phylogenetic and population-based analyses. Until now, there has been no clear indication that one genetic marker can efficiently be used for both purposes. Previously, we had shown that DNA sequence analysis of the protein A gene variable repeat region (*spa* typing) provides a rapid and accurate method to discriminate *Staphylococcus aureus* outbreak isolates from those deemed epidemiologically unrelated. Here, using the hypothesis that the genetic macrovariation within a low-level recombinogenic species would accurately be characterized by a single-locus marker, we tested whether *spa* typing could congruently index the extensive genetic variation detected by a whole-genome DNA microarray in a collection of 36 isolates, which was recovered from 10 countries on four continents over a period of four decades, that is representative of the breadth of diversity within *S. aureus*. Using *spa* and *coa* typing, pulsed-field gel electrophoresis (PFGE), and microarray and multilocus enzyme electrophoresis (MLEE) data in molecular epidemiologic and evolutionary analyses, we determined that *S. aureus* likely has a primarily clonal population structure and that *spa* typing can singly index genetic variation with 88% direct concordance with the microarray and can correctly assign isolates to phylogenetic lineages. *spa* typing performed better than MLEE, PFGE, and *coa* typing in discriminatory power and in the degree of agreement with the microarray at various phylogenetic depths. This study showed that genetic analysis of the repeat region of protein A comprehensively characterizes both micro- and macrovariation in the primarily clonal population structure of *S. aureus*.

Staphylococcus aureus is the leading cause of nosocomial infections (21) and is responsible for a wide range of human diseases, including endocarditis, food poisoning, toxic shock syndrome, septicemia, skin infections, soft tissue infections, and bone infections, as well as bovine and ovine mastitis. The recent emergence of community-associated methicillin-resistant *S. aureus* (MRSA) strains (23) and the movement of the *vanA* operon from *Enterococcus faecalis* into MRSA, establishing strains with high-level vancomycin resistance (3), further heighten the public health concerns. Thus, understanding and controlling the spread of *S. aureus*, in both the hospital and community settings, is now of paramount importance.

S. aureus is a heterogenous (polymorphic) species (13) that was recently found to have a clonal population structure (11). Therefore, it is believed that *S. aureus* does not undergo extensive recombination, diversifies largely by nucleotide mutations, and shows a high degree of linkage disequilibrium (nonrandom associations between genetic loci). In order to distinguish strains within a heterogenous species for local epidemiologic or outbreak investigation purposes, a highly discriminating genetic marker that accumulates variation rapidly, such as pulsed-field gel electrophoresis (PFGE), is required (10). For studying longer-term or global pathogen epidemiology and population genetics, such as the worldwide distribution and frequency of bacterial lineages, virulence properties associated with certain lineages, etc., a highly discriminating genetic marker that accumulates genetic variation relatively slowly is desired (10). For many years multilocus enzyme electrophoresis (MLEE) and, recently, multilocus sequence typing (MLST) have been effectively used for this purpose (17). However, no single technique had been clearly shown to be efficient in both outbreak and global investigations due to the different requirements for rates of accumulating genetic variation.

We previously reported on DNA sequencing of a polymorphic 24-bp variable-number tandem repeat (VNTR) within the 3′ coding region of the *S. aureus*-specific staphylococcal protein A (*spa*) gene for discrimination of *S. aureus* in outbreak settings (30). This comparative study of multiple genotyping methods revealed that *spa* typing is as capable for outbreak purposes as other molecular methods, including PFGE. There are indications that *spa* typing may also be in agreement with MLST (4, 24), which is likely a reflection of the clonal nature of *S. aureus*. If a single-locus DNA sequence-based marker such as *spa* typing was capable of simultaneously indexing genetic variations that accumulate both rapidly and slowly (micro- and macrovariation) by two independent mechanisms,

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then this one marker would be useful in both local and global, long-term epidemiologic and population-based studies. Use of a single-locus marker is also inherently less expensive, time-consuming, and error prone than multilocus techniques, such as MLST (25).

Here we formally investigate whether *spa* typing, in addition to its usefulness in outbreak investigations, also accurately reflects variation occurring throughout the entire chromosome, enabling its use in global population studies and as a simple and rapid way of assigning strains to phylogenetic lineages. In order to test this, we used a collection of 36 strains previously characterized by a DNA microarray based upon over 90% of the genome of the COL strain (13) and compared the results obtained by *spa* typing these strains to the results of the DNA microarray. The 36 strains, which were selected for being representative of the most abundant lineages from over 2,000 spatio-temporally diverse isolates typed by MLEE, provided a highly diverse collection that also enabled detection of the likely population structure of *S. aureus*. Additionally, the strains were analyzed by PFGE and *coa* typing (31) to better assess the discriminatory ability, accuracy, and relative rate of genetic change (clock speed) of *spa* typing.

MATERIALS AND METHODS

Bacterial strains. Thirty-six *S. aureus* strains, as described in the report by Fitzgerald et al. (13), including the fully sequenced COL strain, were analyzed. These strains were selected from 2,077 MLEE-typed isolates recovered from 10 countries on four different continents over a period of four decades as part of a population genetics study, to represent the most abundant lineages and the breadth of genetic diversity of *S. aureus*. The majority of strains were isolated from various human disease conditions and some were from bovine and ovine mastitis cases (13), where the chosen strains ($n = 4$) represented the most abundant lineages of *S. aureus* responsible for these economically important animal conditions. Also included in the sample were 11 strains of phylogenetically diverse MRSA (20) and 14 isolates of electrophoretic type (ET) 234, associated with toxic shock syndrome (TSS) and of diverse gene content (13).

Molecular analysis. *spa* and *coa* typing were performed on each of the isolates as previously described (30, 31). A *spa* type refers to the composition of the VNTRs in the 3′ end of the staphylococcal protein A gene (*spa*). The repeats that define a *spa* type are composed of 24 bp (the only exception being two new repeats found in this study [see Results], which were composed of 21 bp), and a total of 38 repeats have now been identified. Each repeat, designated randomly with letters (A to Z, A2, B2, etc.), varies from the others by at least one point mutation. The different types of organization of a repeat region, ranging from 2 to 16 repeats in length and termed a repeat profile, have defined a total of 386 numeric *spa* types among more than 4,000 *S. aureus* isolates in the Public Health Research Institute Tuberculosis Center repository. The *coa* typing methodology is similar to that for *spa* typing; however, the VNTR region in the coagulase gene consists of 81-bp repeats, and a total of 67 different *coa* repeats have been identified to date.

spa typing and *coa* typing each had one isolate for which additional processing was required. MSA 2389 did not produce a strong *spa* PCR product, but Southern hybridization revealed that the polymorphic region was present. A new forward primer slightly upstream of that normally used was designed (F_2, 5′-GAACAACGTAACGGCTTCATCC-3′) and gave good results, suggesting that mutations in the region of the forward primer were responsible for the problem. The *coa* region of MSA 2335 had a plasmid-encoded arsenic transporter DNA sequence inserted just downstream of the coagulase gene stop codon and just upstream of the reverse primer used in *coa* typing, making it difficult to obtain the complete reverse sequence for this region. A primer was designed close to the insertion, and the reverse DNA sequence was obtained.

As done previously (4, 30), *spa* types with similar repeat profiles were grouped together as part of the same numeric lineage because of sequential point mutations (indicated by specific *spa* repeats) in common. The same was done for similar *coa* types (31), and because of the higher number of extant *coa* repeats compared to *spa* repeats, *coa* lineages were able to be constructed, when deeper phylogenetic classification was sought, from *coa* types with fewer repeats in common compared to *spa* typing. *coa* typing results also served as a comparative secondary marker of *spa* typing lineage assignments. However, *spa* typing lineages were assigned independently of *coa* typing results and vice versa and independently of the previously published DNA microarray results (13) discussed below.

Macrorestriction analysis using PFGE was performed (6). Isolates with patterns up to six bands different are considered possibly related (35) and were grouped together in the same alphabetic lineage, with each unique pattern within a lineage also given a secondary numeric code. Patterns that did not fall into any lineages were identified solely with the numeral 1.

The DNA microarray experiments performed by Fitzgerald et al. (13) assayed for the presence of 2,817 open reading frames (ORFs) within each of the 36 strains used in this study. Only 78% of ORFs were shared by all strains. Hierarchical cluster analysis based on the presence or absence of ORFs was used to construct a dendrogram showing chromosomal relationships among the 36 strains (13). The dendrogram (Fig. 1) indicates relatedness with a scale that specifies the Pearson correlation coefficient for each node (1 = identical and 0 = total independence for a pairwise analysis). In this study, lineages of the dendrogram were assessed at deep phylogenetic levels (i.e., the scale was truncated at lower correlation coefficients, 0.5 and 0.31, forming fewer and fewer genetic groups) and at a more discriminating level (i.e., the scale was truncated at a higher correlation coefficient, 0.81, forming more genetic groups).

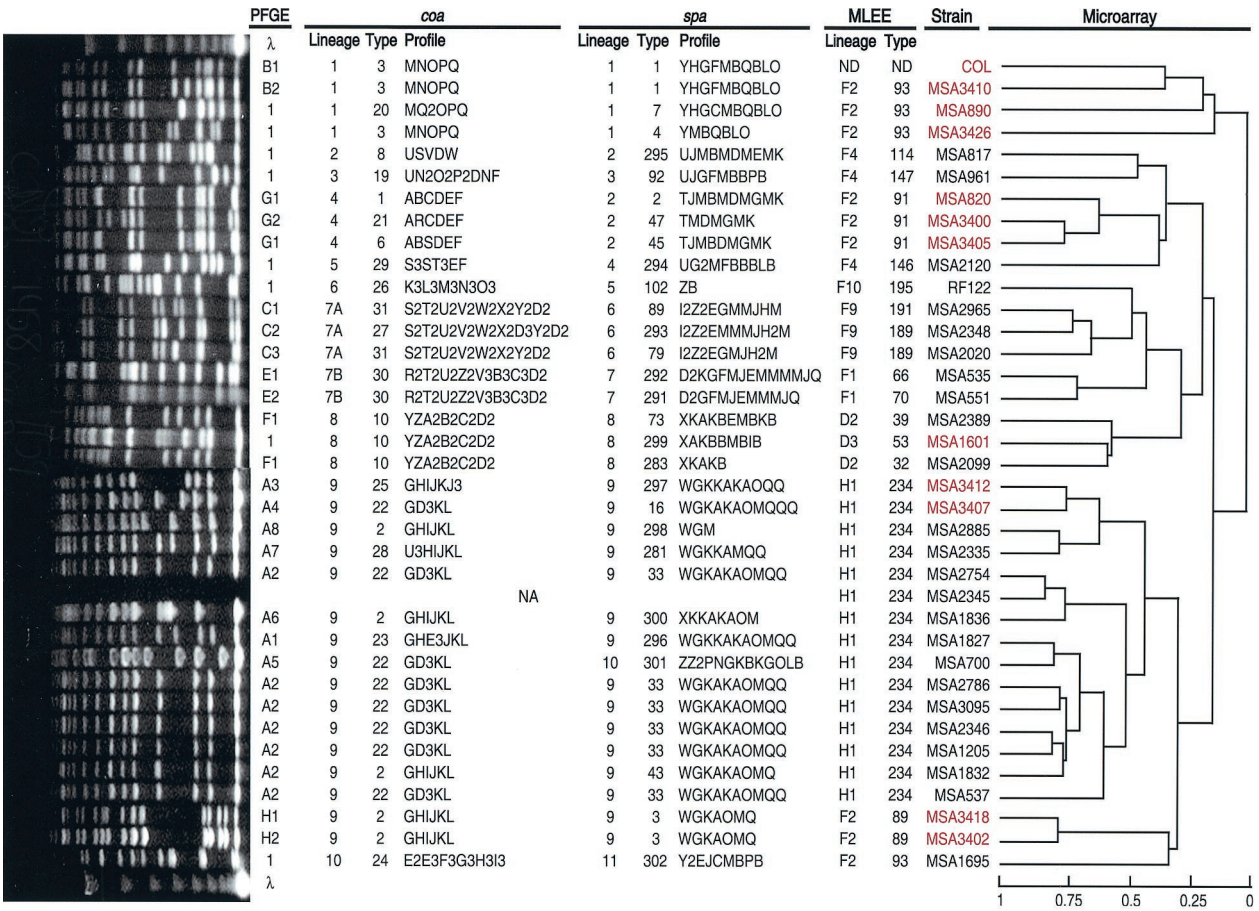
Comparison of genetic markers. The degree of congruence between different typing schemes was determined via cross-classification analysis of all possible pairs of isolates (28, 37). Briefly, for each isolate pair it was determined whether their lineages matched (i.e., were identical) or mismatched (i.e., were different) according to each typing technique. A two-by-two table was constructed for each two-technique comparison, and the percent cell concordance was calculated.

Discriminatory power was measured with Simpson's index of diversity, which calculates the probability that any two isolates will have different genotypes (14). Molecular evolutionary analyses of the *spa* and *coa* repeats were conducted with MEGA version 2.1 (16). The average numbers of synonymous substitutions per synonymous site (dS) and nonsynonymous substitutions per nonsynonymous site (dN) were calculated by using the overall mean Nei-Gojobori (Jukes-Cantor corrected) method (22) with pairwise deletion handling of gaps and standard error determined with 1,000 bootstrap replications. Z-tests to detect selection pressure on the repeats were performed with the null hypothesis $dN = dS$ and three different alternative hypotheses: $dN \neq dS$ (test of neutrality), $dN > dS$ (positive selection), and $dN < dS$ (purifying selection). The PROTPARAM program (http://us.expasy.org/tools/protparam.html) from the Swiss Institute of Bioinformatics ExPASy (Expert Protein Analysis System) proteomics server (1) was used to determine the grand average of hydropathicity index, a measurement of hydrophobicity calculated for the amino acid sequence of each *spa* repeat.

RESULTS

Organization of *spa*, *coa*, and PFGE genotypes and DNA microarray dendrogram. All 36 strains analyzed were typeable by *spa*, *coa*, and PFGE techniques. Figure 1 shows the previously produced dendrogram for the DNA microarray data and the MLEE lineage and ET designations, next to which are the *spa* and *coa* lineage, type, and profile data and the PFGE genotype data for each of the isolates used in this study.

(i) *spa* typing. As described in Materials and Methods and elsewhere (30), a *spa* type is composed of various repeats, each of which represents 24 nucleotides (eight codons). In this study two new *spa* repeats (Y2 and Z2) were found among the isolates, both of which contained never-before-seen single-codon deletions (Fig. 2 lists all *spa* repeats in *S. aureus* that we have identified to date), and 14 new *spa* types were identified (*spa* types 281, 283, and 291 to 302). In total, *spa* typing resolved 29 distinct *spa* types for the 36 strains. The repeat profiles of these *spa* types were organized for the 36 strains into 11 different lineage groups (Fig. 1), 5 of which contained a single strain. Lineages were formed by grouping together strains with similar *spa* repeat profiles, which represents the accumulation of identical point mutations, suggesting a genetic relatedness and descent from a common ancestor (30, 31). The



PFGE	*coa*			*spa*			MLEE		Strain
λ	Lineage	Type	Profile	Lineage	Type	Profile	Lineage	Type	
B1	1	3	MNOPQ	1	1	YHGFMBQBLO	ND	ND	COL
B2	1	3	MNOPQ	1	1	YHGFMBQBLO	F2	93	MSA3410
1	1	20	MQ2OPQ	1	7	YHGCMBQBLO	F2	93	MSA890
1	1	3	MNOPQ	1	4	YMBQBLO	F2	93	MSA3426
1	2	8	USVDW	2	295	UJMBMDMEMK	F4	114	MSA817
1	3	19	UN2O2P2DNF	3	92	UJGFMBBPB	F4	147	MSA961
G1	4	1	ABCDEF	2	2	TJMBMDMGMK	F2	91	MSA820
G2	4	21	ARCDEF	2	47	TMDMGMK	F2	91	MSA3400
G1	4	6	ABSDEF	2	45	TJMBDMGMK	F2	91	MSA3405
1	5	29	S3ST3EF	4	294	UG2MFBBBLB	F4	146	MSA2120
1	6	26	K3L3M3N3O3	5	102	ZB	F10	195	RF122
C1	7A	31	S2T2U2V2W2X2Y2D2	6	89	I2Z2EGMMJHM	F9	191	MSA2965
C2	7A	27	S2T2U2V2W2X2D3Y2D2	6	293	I2Z2EMMMJH2M	F9	189	MSA2348
C3	7A	31	S2T2U2V2W2X2Y2D2	6	79	I2Z2EGMJH2M	F9	189	MSA2020
E1	7B	30	R2T2U2Z2V3B3C3D2	7	292	D2KGFMJEMMMMJQ	F1	66	MSA535
E2	7B	30	R2T2U2Z2V3B3C3D2	7	291	D2GFMJEMMMJQ	F1	70	MSA551
F1	8	10	YZA2B2C2D2	8	73	XKAKBEMBKB	D2	39	MSA2389
1	8	10	YZA2B2C2D2	8	299	XAKBBMBIB	D3	53	MSA1601
F1	8	10	YZA2B2C2D2	8	283	XKAKB	D2	32	MSA2099
A3	9	25	GHIJKJ3	9	297	WGKKAKAOQQ	H1	234	MSA3412
A4	9	22	GD3KL	9	16	WGKAKAOMQQQ	H1	234	MSA3407
A8	9	2	GHIJKL	9	298	WGM	H1	234	MSA2885
A7	9	28	U3HIJKL	9	281	WGKKAMQQ	H1	234	MSA2335
A2	9	22	GD3KL	9	33	WGKAKAOMQQ	H1	234	MSA2754
			NA				H1	234	MSA2345
A6	9	2	GHIJKL	9	300	XKKAKAOM	H1	234	MSA1836
A1	9	23	GHE3JKL	9	296	WGKKAKAOMQQ	H1	234	MSA1827
A5	9	22	GD3KL	10	301	ZZ2PNGKBKGOLB	H1	234	MSA700
A2	9	22	GD3KL	9	33	WGKAKAOMQQ	H1	234	MSA2786
A2	9	22	GD3KL	9	33	WGKAKAOMQQ	H1	234	MSA3095
A2	9	22	GD3KL	9	33	WGKAKAOMQQ	H1	234	MSA2346
A2	9	22	GD3KL	9	33	WGKAKAOMQQ	H1	234	MSA1205
A2	9	2	GHIJKL	9	43	WGKAKAOMQ	H1	234	MSA1832
A2	9	22	GD3KL	9	33	WGKAKAOMQQ	H1	234	MSA537
H1	9	2	GHIJKL	9	3	WGKAOMQ	F2	89	MSA3418
H2	9	2	GHIJKL	9	3	WGKAOMQ	F2	89	MSA3402
1	10	24	E2E3F3G3H3I3	11	302	Y2EJCMBPB	F2	93	MSA1695
λ									

Microarray: 1 0.75 0.5 0.25 0

FIG. 1. Molecular characterization of strains. From right to left: (i) a dendrogram showing the estimated relationships of the 36 strains based on a whole-genome DNA microarray with a scale indicating the Pearson correlation coefficient for each node (0, totally unrelated; 1, identical); (ii) a listing of strains (MSA, Musser *S. aureus*; red, MRSA strains); (iii) MLEE lineage and ET for each strain; (iv) *spa* and *coa* lineages, types, and profiles for each strain (note that *coa* lineages 7A and 7B can be considered separate lineages [lineages I] or together as one lineage [lineages II]); (v) PFGE patterns and types (λ, molecular weight standard of lambda DNA concatemers from New England BioLabs). NA, strain was not available. The dendrogram was adapted from reference 13 with permission of the publisher.

same *spa* profile groupings were obtained by using a global multiple-sequence alignment program (data not shown). As an example, although *spa* type 46 (YMBQBLO) differed in the number of repeats from *spa* type 7 (YHGCMBQBLO), the two were grouped together in *spa* lineage 1 because they contained in common many identical repeats representing exact nucleotide polymorphisms shared by both.

(ii) *coa* typing. Thirty-one new *coa* repeats were found, and 13 new *coa* types were identified (*coa* types 19 to 31). In total, *coa* typing resolved 19 different *coa* types (Fig. 1), which, by using the same methodology as for *spa* typing, could be organized into either 11 or 10 (for deeper phylogenetic classification) lineages, which were designated *coa* lineages I and II, respectively (i.e., by counting *coa* lineages 7A and 7B separately or as one).

(iii) PFGE. A total of 28 different PFGE genotypes were resolved. There were 15 PFGE strain families formed from strains considered identical or possibly related, with up to six band differences present between any two members of a family. Eight of these families had only one isolate and were assigned a unique PFGE type (designated 1). PFGE patterns and types are shown in the left portion of Fig. 1.

(iv) DNA microarray dendrogram. All *spa* and *coa* lineages were compared to the "gold standard" microarray to determine whether agreement exists and the extent of discriminatory and grouping abilities of *spa* and *coa* typing relative to the microarray. Because the microarray distinguished all 36 isolates from one another (13), microarray lineages had to be defined according to correlation coefficient truncation values, which was done in the following way. Moving as deeply into the phylogeny as possible on the DNA microarray dendrogram (shown on the right side of Fig. 1), at a correlation coefficient of <0.25, two major clusters are distinguished, one containing 4 closely related strains that were always grouped together as one lineage and the other containing the remaining 32 genetically diverse strains that were grouped at three different levels. Assessment of these 32 strains at a very deep phylogenetic correlation coefficient of 0.31 classified them into only four lineages, creating for the 36 strains a total of five DNA microarray lineages, which hereafter will be referred to as the

1	2	3	4	5	6	7	8	***spa* Repeat Code**
AAA	GAA	GAC	AAC	AAC	AAA	CCT	GGC	D1
AAA	GAA	GAC	AAC	AAA	AAA	CCT	GGC	A1
AAA	GAA	GAC	AAT	AAC	AAG	CCT	GGC	H1
AAA	GAA	GAC	AAC	AAC	AAG	CCT	GGC	F1
AAA	GAA	GAC	AAC	AAA	AAG	CCT	GGC	C1
AAA	GAA	GAC	AAC	AAA	AAG	CCT	AGC	F2*
AAA	GAA	GAC	AAC	AAA	AAA	CCT	GGT	B1
AAA	GAA	GAC	AAC	AAC	AAA	CCT	GGT	E1
AAA	GAA	GGC	AAC	AAA	AAA	CCT	GGT	I1*
AAA	GAA	GAC	AAC		AAG	CCT	GGT	Z2+
AAA	GAA	GAC	AAC	AAC	AAG	CCT	GGT	G1
AAA	GAA	GAC	AAT	AAC	AAG	CCT	GGT	C2
AAA	GAA	GAC	AGC	AAC	AAG	CCT	GGC	E2
AAA	GAA	GAC	GGC	AAC	AAA	CCT	GGC	J1
AAA	GAA	GAC	GGC	AAA	AAA	CCT	GGC	G2
AAA	GAA	GAT	GGC	AAC	AAA	CCT	GGC	N1
AAA	GAA	GAC	GGC	AAC	AAG	CCT	GGC	L1
AAA	GAA	GAT	GGC	AAC	AAG	CCT	GGC	P1
AAA	GAA	GAT	GGT	AAC	AAA	CCT	GGC	R1
AAA	GAA	GAT	GGC	AAC	AAA	CCT	GGT	O1
AAA	GAA	GAC	GGC	AAC	AAA	CCT	GGT	K1
AAA	GAA	GAC	GGC	AAC	AAG	CCT	GGT	M1
AAA	GAA	GAT	GGC	AAC	AAG	CCT	GGT	Q1
AAA	GAA	GAT	GGC	AAC	AAG	CCT	AGT	H2*
AAA	GAA	GAT	AAC	AAC	AAG	CCT	GGT	B2*
CAA	GAA	GAC	GGC	AAC	AAG	CCT	GGT	U2+
CAA	GAA	GAC	AAC	AAC	AAG	CCT	GGT	V2+
GAG	GAA	GAC	AAC	AAC	AAA	CCT	GGC	I2*
GAG	GAA	GAC	AAC	AAA		CCT	GGC	Y2+
GAG	GAA	GAC	AAT	AAC	AAG	CCT	GGC	Y1
GAG	GAA	GAC	AAC	AAC	AAG	CCT	GGC	W1
GAG	GAA	GAC	AAC	AAC	AAG	CCT	AGC	V1
GAG	GAA	GAC	AAC	AAA	AAA	CCT	GGT	T1
GAG	GAA	GAC	AAC	AAC	AAA	CCT	GGT	U1
GAG	GAA	GAC	AAT	AAC	AAA	CCT	GGT	D2
GAG	GAA	GAC	AAT	AAC	AAG	CCT	GGT	Z1
GAG	GAA	GAC	AAC	AAC	AAG	CCT	GGT	X1
GAG	GAA	GAC	GGC	AAC	AAA	CCT	GGT	A2

FIG. 2. DNA sequences for the 38 *spa* repeats identified to date in *S. aureus.* Individual repeat codes are shown on the right. Sequences are organized and displayed as eight colored codons, and codons in the same column with the same color represent the same three nucleotides. Asterisks indicate repeats that have been redefined, and crosses indicate new repeats found since those previously reported (30).

0.31 microarray lineages. Assessment of the 32 strains at a slightly less deep correlation coefficient of 0.5 produced a total of 12 lineages (named 0.5 microarray lineages) for the 36 strains, and when truncation was at a discriminating 0.81 value, 35 groups were created for the 36 strains (named 0.81 microarray groups). The 0.5 depth was chosen to create microarray lineages after exploring where on the microarray dendrogram the upper limit of *spa* typing's ability was to group strains together into lineages, beyond which it could not group strains further. The 0.81 truncation value on the microarray dendrogram was the upper limit of *spa* typing's ability to discriminate strains, beyond which it was unable to distinguish related strains further. Lastly, the 0.31 value was the deepest phylogenetic level on the microarray dendrogram at which *coa* typing (number of *coa* lineages at this level = 10) could group strains together into lineages.

Congruence between genetic markers. The 11 different *spa* typing lineages were 88% concordant with the 12 0.5 microarray lineages in cross-classification analysis of all possible pairs of isolates based on matched or mismatched lineages (Table 1). Therefore, any two strains determined to be either of the same lineage or from different lineages by one technique were given an identical designation by the other technique 88% of the time. Other concordance values are listed in Table 1, which shows that *spa* typing is slightly more congruent with the DNA microarray (88%) than is MLEE (87%). Also, *spa* typing and MLEE were 87% congruent in designating lineages. Only one strain, MSA 700 (*spa* type 301), had a *spa* profile that was in no way allied with those of closely related strains as determined by the DNA microarray (this strain was, however, correctly classified by *coa* typing). Overall, these results show that *spa* typing, which relies on a single VNTR locus, can serve as a marker of deeper phylogeny and is congruent with the DNA microarray, which detects genome-scale variation.

coa lineages I were highly concordant with the 0.5 microarray lineages (89%), and lineages II were even more so with the 0.31 microarray lineages (95%) of the microarray. MLEE lineages had lower agreement than *coa* typing did with the 0.5 and 0.31 microarray lineages (87 and 85%, respectively). Lastly, *coa* lineages were highly concordant with MLEE lineages (90%), and *coa* types were highly concordant with MLEE types (87%).

TABLE 1. Cross-classification concordance results

Typing technique	% Concordance[a] between:			
	Microarray	MLEE	PFGE	*coa*
spa	88 (lineages/0.5), 97 (types/0.81)	87 (lineages/lineages)	98 (types/types)	97 (lineages/lineages I)
coa	89 (lineages I/0.5), 95 (lineages II/0.31)	90 (lineages I/lineages), 87 (types/types)		
MLEE	87 (lineages/0.5), 85 (lineages/0.31), 83 (types/0.81)			

[a] The percent of direct concordance between any two typing schemes is shown where the two intersect on this matrix. A (row/column) descriptor follows each concordance value, as follows. For microarray, 0.31 indicates 0.31 microarray lineages, 0.5 indicates 0.5 microarray lineages, and 0.81 indicates 0.81 microarray groups. For *spa* typing, lineages and types were separately analyzed. For *coa* typing, lineages and types were separately analyzed; lineage I includes 11 *coa* lineages produced by counting *coa* lineages 7A and 7B separately, and lineage II includes 10 *coa* lineages produced by counting *coa* lineages 7A and 7B as one lineage for deeper phylogenetic analysis. For MLEE, lineages and types were separately analyzed. For PFGE, individual types were analyzed. As an example, the concordance between *spa* typing and the microarray is 88% for *spa* typing lineages and the 0.5 microarray lineages.

Discriminatory power. The DNA microarray, which assayed for the presence of >2,800 ORFs, was able to differentiate all 36 isolates from one another. The results obtained by Fitzgerald et al. (13) showed high variability, up to 22%, in genetic content among different strains and demonstrated that no two strains were identical. Simpson's index of diversity calculates the probability that two randomly selected isolates will have different genotypes, and therefore the DNA microarray had a 100.0% resolving power. There were 29 different *spa* types, and the probability that any two isolates could be differentiated based on *spa* typing was 97.3%, which was higher than the values for MLEE (81.6%), *coa* typing (92.7%), and PFGE (96.4%) (Table 2). Fourteen of the isolates were members of a TSS clone, classified as identical by MLEE (ET 234) and very closely related by the DNA microarray, and the slightly lower resolution of *spa* typing in comparison to the microarray was largely due to difficulty in resolving differences among the members of this group. However, the nine different *spa* types resolved for this group were greater than the number of types distinguished by PFGE ($n = 8$), *coa* typing ($n = 5$), or MLEE ($n = 1$). The combination of *spa* typing and PFGE resulted in only a 0.3% increase in resolving power over *spa* typing alone. A summary of the genetic diversity detected by various techniques is given in Table 2. *coa* typing had approximately 5% less resolving power than *spa* typing, and together with its strong congruence with the microarray at deep phylogenetic levels and with *spa* typing (see next section), *coa* performed as a secondary marker of lineage and as a verifier of *spa* typing groupings.

The dendrogram produced by the DNA microarray, at a correlation coefficient of 0.81, split the 36 strains into 35 different groups (i.e., only two strains shared the same designation). At this highly discriminating level, individual *spa* types ($n = 29$) for the 36 isolates were 97% concordant with the DNA microarray in cross-classification of the isolates. However, MLEE types were only 83% concordant with the microarray at this level. Additionally, using this same method of analysis, individual *spa* and PFGE types were 98% concordant. These data, along with *spa* typing's capability of resolving >3 trillion potential *spa* types (see below), illustrate the very high discriminatory ability of *spa* typing and its congruence with other high-resolution techniques, such as the microarray and PFGE, for a spatio-temporally diverse collection of isolates.

Linkage disequilibrium and a clonal population structure. The performance of *coa* and *spa* typing on all isolates aided in determining the presence of linkage disequilibrium in this diverse set of methicillin-susceptible *S. aureus* and MRSA isolates. Only two isolates had a *spa* type or *coa* type discrepancy (MSA 700 and MSA 817, respectively) in the expected agreement of typing results between the two techniques based on groupings of isolates in Fig. 1. In cross-classification analysis of lineages produced by *spa* and *coa* typing, the two techniques were 97% concordant (Table 1). In order to assess the significance of these findings, their probability of occurrence by chance was determined. There were an average of 9 repeats per *spa* type, and since there are 38 different *spa* repeats, 11 of which can be present only as the first repeat, a total of $27^8 \times 11$ or >3 trillion different *spa* types can potentially be resolved. Likewise, there were an average of 6 *coa* repeats per *coa* type and there are 67 different *coa* repeats, with 11 present only in the first position, producing a total of >6 billion possible *coa* types. The product of the number of types that *spa* and *coa* typing can each resolve equals 1.9×10^{22} and represents the total possible number of *spa* and *coa* allelic profiles that the two techniques in combination can resolve. Therefore, the probability that isolates with a related *spa* type would happen to have a related *coa* type by chance is practically zero. (This finding is made even more conclusive because it is unlikely, based on their approximately 100-kb distance from one another, that the *spa* and *coa* genes could be inherited together in a single transfer event [30].)

Whereas *spa* and *coa* typing each index variation at single loci, the DNA microarray, MLEE, and PFGE index variation throughout the chromosome, and detecting agreement with these latter techniques is a particularly effective way of demonstrating linkage disequilibrium because they are such highly informative markers. The strong agreement of *spa* typing with PFGE and of *spa* typing, *coa* typing, and MLEE each with one

TABLE 2. Genetic diversity of typing schemes

Technique(s)	No. of genotypes	Index of diversity (%)
Microarray	36	100.0
spa typing	29	97.3
PFGE	28	96.4
coa typing	19	92.7
MLEE	15	81.6
spa and PFGE	31	97.6
spa and *coa*	29	97.3
spa and MLEE	29	97.3

another and with the DNA microarray at various phylogenetic depths (Table 1) highlights the presence of nonrandom associations among loci throughout the genome and the presence of a phylogenetic signal in the isolates. Furthermore, agreement of phylogenetic lineage assignment results from single-locus and other molecular techniques indicates low levels of recombination (27) within this population of isolates. Such widespread linkage disequilibrium among various independent genetic markers and low levels of recombination are strong evidence (7, 34) that *S. aureus* has a primarily clonal population structure.

Evolutionary pressure on repeats. In addition to analysis based on DNA sequence data from the *spa* locus, the amino acid profile of the *spa* repeats for each isolate was analyzed. Amino acid typing results, unlike those produced from DNA sequences, were unable to be organized in any particular way, as they were all similar, demonstrating that the single-nucleotide polymorphisms within the polymorphic region throughout even distant related lineages are usually silent. This conservation of amino acids revealed interesting structural patterns; for example, nearly all repeat profiles began with the same eight amino acids and ended with the same eight amino acids, although major variation exists at the DNA level. Consistent with these results were findings that all 38 different *spa* repeats identified thus far had similar amino acid hydropathicity indices (range, −3.0 to −2.5) and similar peptide secondary structures (data not shown). For the 38 *spa* repeats, the dS/dN value, a ratio of the number of synonymous substitutions per potential synonymous site to number of nonsynonymous substitutions per potential nonsynonymous site, was 6.4 (a ratio of <1 indicates positive selection, a ratio of 1 indicates no selection pressure [i.e., neutral evolution], and a ratio of >1 indicates purifying selection). The dS value was 0.72 (standard error, 0.15), and the dN value was 0.11 (standard error, 0.06). The dS/dN ratio for the 30 *spa* repeats found in this collection similarly equaled 6.5. Furthermore, analysis of the eight individual codons that comprise the *spa* repeat region from all 38 repeats revealed that no codon had a low dS/dN value, ruling out the possibility that a certain part of a repeat might be under positive selection but was undetected. Lastly, a Z-test for detecting purifying selection was significant for the *spa* repeats ($P = 0.0004$). For the 67 *coa* repeats identified thus far, the dS/dN ratio was 3.8 and was identical to the ratio for the 60 *coa* repeats found in this collection. Therefore, the *coa* repeat region is also highly conserved ($P = 0.009$ for the Z-test of purifying selection). The lack of positive selection and the presence of strong purifying selection precludes the polymorphic *spa* and *coa* loci from accumulating variation linked to outside sources, and instead they reflect variation produced intrinsically by the organism, allowing them to be genetic markers for global epidemiologic studies.

DISCUSSION

The recent advances in bacterial genomics, bioinformatics, and DNA sequencing have enriched the molecular tools of population geneticists, evolutionary biologists, and hospital infection control teams tracking suspected outbreaks. Historically MLEE, and now MLST, have been the most commonly used methods for population geneticists and those studying global epidemiology because these techniques accumulate genetic variation slowly; PFGE has been preferred for outbreak investigations because it accumulates variation rapidly; and, depending on whether micro- or macroevolutionary insights are sought, evolutionary biologists may use either approach. Comparative genomics and DNA microarrays are emerging as two useful platforms for molecular population and evolutionary biologists to discriminate phylogenies based on genome-scale variation (2, 13, 19, 29, 33). In this study we showed that sequencing of a single polymorphic VNTR locus, already proven to be effective for use in outbreak settings, produces results strongly congruent with that of a whole-genome DNA microarray for a collection of geographically and temporally diverse isolates. Unlike more expensive and labor-intensive techniques such as MLST (25), which requires sequencing approximately 3,500 bp from seven different loci per isolate, *spa* typing is based on the sequencing of only a single PCR amplicon that ranges in size from 200 to 600 bp. Furthermore, it should be noted that MLST is not able to distinguish various MRSA strains (9) and, unlike *spa* typing, has not been tested and shown to be successful in an outbreak setting where endemic clones require high levels of discrimination.

In global population studies a genetic marker that can distinguish strains at a fine resolution is needed to discern subtle variation and concurrently link strains together into broad phylogenetic groups to study phenomena such as the nonrandom distribution of virulence among bacterial lineages. The DNA microarray met these criteria because it differentiated all 36 strains and, because it found that all of the strains had 2,198 ORFs in common, was able to generate a dendrogram displaying deep phylogenetic relationships among strains from very distant lineages. In its most discriminatory capacity, i.e., at the level of individual *spa* types, *spa* typing can differentiate strains comparably to the whole-genome microarray at the 0.81 level of the dendrogram, which splits 36 isolates into 35 different groups. Yet *spa* typing, when used at the level of *spa* lineages, is able to assign strains to phylogenetic lineages and group strains comparably to the microarray at the 0.5 level. Moreover, *spa* typing was tolerant of mutations and repeat insertion-deletion events without compromising its ability to assign strains to lineages. This attribute also minimizes compounding the effects of minor sequencing errors when assigning genotypes, as has been problematic elsewhere (5).

Based on the results shown here and elsewhere (30, 31) and on the thousands of *S. aureus* isolates in our collection that have been *spa* typed, it is evident that recombination events involving the *spa* locus, which might reduce *spa* typing accuracy, take place very rarely. If verification of a *spa* lineage was desired, then MLST or DNA microarray analysis of certain representative strains would be appropriate. Concern over *spa* typing lacking the ability to discern differences among closely related strains, such as the ET 234 TSS strains of this study, could be resolved with a highly evolving marker. Our recent study demonstrated that sequence analysis of a locus containing a repeat encoding a serine-aspartate dipeptide is capable of distinguishing among strains with identical *spa* genotypes (L. Koreen, S. Ramaswamy, S. Naidich, E. A. Graviss, and B. Kreiswirth, Abstr. 103rd Gen. Meet. Am. Soc. Microbiol., abstr. C-415, 2003). It is also important to note that *spa* typing has long-term in vitro and in vivo stability (30), and every one of

the thousands of *S. aureus* isolates that we have attempted to *spa* type was typeable. Use of the alternative primer used in this study for MSA 2389 is suggested in the very rare instances of no *spa* amplification (8, 26), which are likely due to sequence mutations in the primer region.

In order to understand why *spa* typing is so highly discriminating, it is important to analyze two major contributory components of discriminatory power in a sequence-based VNTR typing system: the background rate of nucleotide mutation within the repeats and the extent of repeat number variation (i.e., variability in repeat region size). According to the neutral theory of evolution, the number of synonymous substitutions per synonymous site (dS) is proportional to the rate of nucleotide mutation of a gene (15). The dS value for *coa* was 0.48, indicating that the *spa* region (dS = 0.72) has an approximately 24% higher background rate of mutation than *coa*. This alone does not explain the superior discriminatory power of *spa* over *coa* typing, since individual *spa* repeats are usually 57 bp shorter than *coa* repeats and thus the number of potential synonymous mutation sites is much less in *spa* repeats. Calculations based only on mutation rate showed that *coa* typing would theoretically be more discriminating than *spa* typing (data not shown). However, when considering only variation in the number of repeats present among isolates, for the average *spa* lineage where more than one isolate was present, 73% of isolates varied in number of repeats, compared with just 37% for *coa* typing. Therefore, typing with *spa* generated nearly twice the variation in repeat region sizes among related isolates than did typing with *coa*. When taking into consideration all factors contributing to discriminatory power, *spa* typing was able to distinguish, for the average group of related isolates, 89% of isolates, versus 55% for *coa* typing (versus 100% for the DNA microarray), and thus produced about 1.6 times as much variability as did *coa* typing. It is likely that via slipped-strand mispairing (36), the *spa* repeats are more prone to duplication and deletion because they are smaller than the *coa* repeats. This along with a large dS value contributes to the high discriminatory ability of *spa* typing.

The congruence among *spa* typing, *coa* typing, PFGE, MLEE, and the DNA microarray indicates low levels of recombination, a predominantly clonal population structure, and the presence of a phylogenetic signal within *S. aureus*. A clonal population structure, as opposed to one of panmixia caused by high rates of recombination, exists when strain progeny diversify from their ancestors mainly through nucleotide mutations. Epidemic spread of a highly recombinogenic species in linkage equilibrium may appear to be clonal (32); however, in this study such a structure was ruled out because of the representative nature of the strain collection. Although the majority of isolates used in this study were disease causing, they were selected from over 2,000 disease and carriage isolates as representing the most abundant lineages and, as shown by the microarray data, certainly contained great diversity. In addition, recent evidence points towards disease-causing populations of *S. aureus* being either as diverse or even more diverse than carriage populations (11, 25). Previous findings based on MLST showing that *S. aureus* was highly recombinogenic (5, 12) have since been amended due to sequencing errors (5). The revised data set (11) demonstrated that *S. aureus* has a predominately clonal population structure, and our findings corroborate this result.

It is evident that repeat composition and organization, and not the number of repeats, allow *spa* typing to correlate with the DNA microarray data. Thus, primary assignment of lineages is via nucleotide mutation, followed by intralineage variation via addition and deletion of repeats, suggesting that point mutations occur at a much lower rate (clock speed) than repeat number variation. Therefore, through the use of slow point mutations and fast-occurring changes in the number of repeats, *spa* typing solves the longstanding impasse in finding one technique that is capable of accumulating genetic variation both as slowly as the major chromosomal changes that distinguish lineages occur and rapidly, at two independent levels, enabling it to be used effectively in answering long- and short-term epidemiologic questions, respectively.

The strong congruence of *spa* typing's genotyping results with multiple techniques implies that the single-nucleotide polymorphisms within the repeat region occurred only once, as recombination and repeated mutation would obliterate any compatibility (27). Occurrence of neutral (as evidenced by the dS/dN analysis) mutations due to purifying selection just once at this locus, probably resulting from functional biological constraints placed on the protein A gene (as opposed to a gene under positive selection or nonexpressed intergenic regions, pseudogenes, etc., under no constraints), along with a clonal population structure within the species, explains how the *spa* locus alone can accurately identify strains throughout the breadth of the species and then correctly assign them to lineages. These factors, along with the higher rate of nucleotide substitutions and greater variation in repeat number compared with the larger-sized *coa* repeats, are criteria that give *spa* typing the capability of reflecting extensive genome-wide variation within *S. aureus*. Using these characteristics as guidelines, we are currently studying single-locus VNTRs for dual use in local and global epidemiologic analyses of other bacteria, such as *E. faecalis* and *Acinetobacter baumannii*, and we predict that in species with known heterogenous clonal population structures, such as *Escherichia coli* (18), suitable single-locus markers should be successful. The relatively new ability to analyze and search genomes for polymorphic repeat regions combined with the unique ability of certain minisatellite VNTRs to display two different levels of genotypic information will provide many attractive candidates for use for a variety of strain typing goals and should be studied for all types of microorganisms.

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